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(57) Abstract

The invention provides strategy for suppressing expression of an endogenous gene, wherein said strategy comprises providing suppression effectors able to bind to the non-coding regions of a gene to be suppressed, to prevent the functional expression thereof. The suppression effectors may be antisense nucleic acids, and the non-coding regions can include the transcribed but non-translated regions of a gene. The strategy can also introduce a replacement gene.

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STRATEGY FOR SUPPRESSING THE EXPRESSION OF AN ENDOGENEOUS GENE BY USING COMPOUNDS THAT ARE ABLE TO BIND TO THE NON-CODING REGIONS OF THE GENE TO BE SUPPRESSED

Field of the Invention

The present invention relates to a strategy and medicaments for suppressing a gene. In particular the invention relates to the suppression of mutated genes which give rise to a dominant or deleterious effect either monogenically or polygenically. The invention relates to a strategy for suppressing a gene or disease allele such that (if required) a replacement gene, gene product or alternative gene therapy can be introduced.

The invention also relates to a medicament or medicaments for use in suppressing a gene or disease allele which is present in a genome of one or more individuals or animals. The said medicament(s) may also introduce the replacement gene sequence, product or alternative therapy.

Generally the strategy of the present invention will be useful where the gene, which is naturally present in the genome of a patient, contributes to a disease state. Generally, the gene in question will be mutated, that is, will possess alterations in its nucleotide sequence that affect the function or level of the gene product. For example, the alteration may result in an

altered protein product from the wild type gene or altered control of transcription and processing. Inheritance or the somatic acquisition of such a mutation can give rise to a disease phenotype or can predispose an individual to a disease phenotype. However the gene of interest could also be of wild type phenotype, but contribute to a disease state in another way such that the suppression of the gene would alleviate or improve the disease state.

Background of the Invention

Studies of degenerative hereditary ocular conditions, including Retinitis Pigmentosa (RP) and various macular dystrophies have resulted in a substantial elucidation of the molecular basis of these debilitating human eye disorders. In a collaborative study, applying the approach of genetic linkage, two x-linked RP genes were localised to the short arm of the X chromosome (Ott et al. 1990). In autosomal dominant forms of RP (adRP) three genes have been localised. The first adRP gene mapped on 3q close to the gene encoding the photoreceptor specific protein rhodopsin (McWilliam et al. 1989; Dryja et al. 1990). Similarly, an adRP gene was placed on 6p close to the gene encoding the photoreceptor specific protein peripherin/RDS (Farrar et al. 1991a,b; Kajiwara et al. 1991). A third adRP gene mapped to 7q (Jordan et al. 1993); no known candidate genes for RP reside in this region of 7q. In addition, the disease gene segregating in a Best's macular dystrophy family was placed on 11q close to the region previously shown to be involved in some forms of this dystrophy (Mansergh et al. 1995). Recently, an autosomal recessive RP gene was placed on 1q (Van Soest et al. 1994). Genetic linkage, in combination with techniques for rapid mutational screening of candidate genes, enabled subsequent identification of causative mutations in the genes ncoding rhodopsin and

peripherin/RDS proteins. Globally about 100 rhodopsin mutations have now been found in patients with RP or congenital stationary night blindness. Similarly about 40 mutations have been characterised in the peripherin/RDS gene in patients with RP or with various macular dystrophies.

Knowledge of the molecular aetiology of some forms of human inherited retinopathies has stimulated the establishment of methodologies to generate animal models for these diseases and to explore methods of therapeutic intervention; the goal being the development of treatments for human retinal diseases (Farrar et al. 1995). Surgical procedures enabling the injection of sub-microlitre volumes of fluid intravitinally or subretinally into mouse eyes have been developed by Dr Paul Kenna. In conjunction with the generation of animal models, optimal systems for delivery of gene therapies to retinal tissues using viral (inter alia Adenovirus, Adeno Associated Virus, Herpes Simplex Type 1 Virus) and non-viral (inter alia liposomes, dendrimers) vectors alone or in association with derivatives to aid gene transfer are being investigated.

Generally, gene therapies utilising both viral and non-viral delivery systems have been applied in the treatment of a number of inherited disorders; of cancers and of some infectious disorders. The majority of this work has been undertaken on animal models, although, some human gene therapies have been approved. Many studies have focused on recessively inherited disorders, the rationale being, that the introduction and efficient expression of the wild type gene may be sufficient to result in a pr vention/amelioration of disease phenotype. In contrast gene therapy for

dominant disorders will require the suppression of the dominant disease allele. Notably the majority of characterised mutations that cause inherited retinal degenerations such as RP are inherited in an autosomal dominant fashion. Indeed there are over 1,000 autosomal dominantly inherited disorders in man. In addition there are many polygenic disorders due to the co-inheritance of a number of genetic components which together give rise to a disease phenotype. Effective gene therapy in dominant or polygenic disease will require suppression of the disease allele while in many cases still maintaining the function of the normal allele.

Strategies to differentiate between normal and disease alleles and to selectively switch off the disease allele using suppression effectors inter alia antisense DNA/RNA, ribozymes or triple helix DNA, targeted towards the disease mutation may be difficult in many cases and impossible in others - frequently the disease and normal alleles may differ by only a single nucleotide. For example, the disease mutation may not occur at a ribozyme cleavage site. Similarly the disease allele may be difficult to target specifically by antisense DNA/RNA or triple helix DNA if there are only small sequence differences between the disease and normal alleles. A further difficulty inhibiting the development of gene therapies is the heterogeneous nature of some dominant disorders - many different mutations in the same gene give rise to a similar disease phenotype. The development of specific gene therapies for each of these would be extremely costly. To circumvent the dual difficulties associated with specifically targeting the disease mutation and the genetic heterogeneity present in some inherited disorders, the present invention aims to provide a

novel strategy for gene suppression and replacement exploiting the noncoding and control regions of a gene.

Suppression effectors have been used previously to achieve specific suppression of gene expression. Antisense DNA and RNA has been used to inhibit gene expression in many instances. Many modifications, such as phosphorothioates, have been made to antisense oligonucleotides to increase resistance to nuclease degradation, binding affinity and uptake (Cazenave et al. 1989; Sun et al. 1989; McKay et al. 1996; Wei et al. 1996). In some instances, using antisense and ribozyme suppression strategies has ~~led~~ led to the reversal of the tumour phenotype by greatly reducing the expression of a gene product or by cleaving a mutant transcript at the site of the mutation (Carter and Lemoine 1993; Lange et al. 1993; Valera et al. 1994; Dosaka-Akita et al. 1995; Feng et al. 1995; Quattrone et al. 1995; Ohta et al. 1996). For example, neoplastic reversion was obtained using a ribozyme targeted to the codon 12 H-ras mutation in bladder carcinoma cells (Feng et al. 1995). Ribozymes have also been proposed as a means of both inhibiting gene expression of a mutant gene and of correcting the mutant by targeted trans-splicing (Sullenger and Cech 1994; Jones et al. 1996). Ribozymes can be designed to elicit autocatalytic cleavage of RNA targets. However the inhibitory effect of some ribozymes may be due in part to an antisense effect of the variable antisense sequences flanking the catalytic core which specify the target site (Ellis and Rodgers 1993; Jankowsky and Schwenzer 1996). Ribozyme activity may be augmented by the use of non-specific nucleic acid binding proteins or facilitator oligonucleotides (Herschlag et al. 1994; Jankowsky and Schwenzer 1996). Triple helix approaches have also been investigated for sequence sp cific gene

suppression - triplex forming oligonucleotides have been found in some cases to bind in a sequence specific manner (Postel et al. 1991; Duval-Valentin et al. 1992; Hardenbol and Van Dyke 1996; Porumb et al. 1996). Similarly peptide nucleic acids have been shown in some instances to inhibit gene expression (Hanvey et al. 1992; Knudson and Nielsen 1996). Minor groove binding polyamides have been shown to bind in a sequence specific manner to DNA targets and hence may represent useful small molecules for future suppression at the DNA level (Trauger et al. 1996). In addition, suppression has been obtained by interference at the protein level using dominant negative mutant peptides and antibodies (Herskowitz 1987; Rimsky et al. 1989; Wright et al. 1989). In some cases suppression strategies have lead to a reduction in RNA levels without a concomitant reduction in proteins, whereas in others, reductions in RNA levels have been mirrored by reductions in protein levels.

The present invention aims to circumvent the shortcomings in the prior art by using a two step approach for suppression and replacement.

According to the present invention there is provided a strategy for suppressing expression of an endogenous gene, wherein said strategy comprises providing suppression effectors able to bind to the non-coding regions of a gene to be suppressed, to prevent the functional expression thereof. Preferably the suppression effectors are antisense nucleic acids. Preferably the targetted non-coding regions include the transcribed but non-translated regions of a gene.

Generally the term suppression effectors includes nucleic acids, peptide nucleic acids (PNAs) or peptides

which can be used to silence or reduce gene expression in a sequence specific manner.

The antisense nucleic acids can be DNA or RNA, can be directed to 5′ and/or 3′ untranslated regions and/or to introns and/or to control regions or to any combination of such untranslated regions. However targetted the binding of the antisense nucleic acid prevents or lowers the functional expression of the endogenous gene. Chimeric antisense nucleic acids including a small proportion of translated regions of a gene can be used in some cases to help to optimise suppression. Likewise Chimeric antisense nucleic acids including a small proportion of promoter regions of a gene can be used in some cases to help to optimise suppression.

Generally the term 'functional expression' means the expression of a gene product able to function in a manner equivalent to or better than a wild type product. In the case of a mutant gene 'functional expression' means the expression of a gene product whose presence gives rise to a deleterious effect.

In a particular embodiment of the invention the strategy further employs ribozymes. These can be designed to elicit cleavage of target RNAs.

The strategy further employs nucleotides which form triple helix DNA.

Nucleic acids for antisense, ribozymes and triple helix may be modified to increase stability, binding efficiencies and uptake as discussed earlier. Nucleic acids can be incorporated into a vector. Vectors include DNA plasmid vectors, RNA or DNA virus vectors. Th se can be combined with lipids, polymers or other

derivatives to aid gene delivery and expression.

The invention further provides the use of antisense nucleotides, ribozymes, triple helix nucleotides or other suppression effectors alone or in a vector or vectors, wherein the nucleic acids are able to bind specifically to untranslated regions of a gene such as the 5′ and 3′ UTRs to prevent the functional expression thereof, in the preparation of a medicament for the treatment of an autosomal dominant disease.

In a further embodiment the non-coding regions of the gene can include promoter regions which are untranslated.

According to the present invention there is provided a strategy for suppressing an endogenous gene and introducing a replacement gene, said strategy comprising the steps of:

1. providing antisense nucleic acid able to bind to at least one non-coding or untranslated region of a gene to be suppressed and

2. providing genomic DNA or cDNA encoding a replacement gene sequence,

wherein the antisense nucleic acid is unable to bind to equivalent non-coding or untranslated regions in the genomic DNA or cDNA to prevent expression of the replacement gene sequence.

The replacement nucleic acids will not be recognised by the suppression nucleic acid. The control sequences of the replacement nucleic acid may belong to a different mammalian species, may belong to a different human gene

or may be similar but altered from those in the gene to be suppressed and may thus permit translation of the part of the replacement nucleic acid to be initiated.

By control sequences is meant sequences which are involved in the control of gene expression or in the control of processing and/or sequences present in mature RNA transcripts and/or in precursor RNA transcripts, but not including protein coding sequences.

In a particular embodiment of the invention there is provided a strategy for gene suppression targeted towards the non-coding regions of a gene and using a characteristic of one of the alleles of a gene, for example, the allele carrying a disease mutation. Suppressors are targeted to non-coding regions of a gene and to a characteristic of one allele of a gene such that suppression in specific or partially specific to one allele of the gene. The invention further provides for replacement nucleic acids containing altered non-coding sequences such that replacement nucleic acids cannot be recognised by suppressors which are targeted towards the non-coding regions of a gene. Replacement nucleic acids provide the wild type or an equivalent gene product but are protected completely or in part from suppression effectors targeted to non-coding regions.

In a further embodiment of the invention there is provided replacement nucleic acids with altered non-coding sequences such that replcement nucleic acids cannot be recognised by naturally occurring endogenous suppressors present in one or more individuals, animals or plants. Replacement nucleic acids with altered non-coding sequences provide the wild type or equivalent

gene product but are completely or partially protected from suppression by naturally occurring endogenous suppression effectors.

In an additional embodiment of the invention there is provided replacement nucleic acids with altered non-coding sequences such that replacement nucleic acids provide a wild type or equivalent gene product or gene product with beneficial characteristics. For example, the 3' non-coding sequences of the replacement nucleic acids could be altered to modify the stability and turn over the RNA expressed from the replacement nucleic acids thereby sometimes affecting levels of ~~resulting~~ resulting gene product.

The invention further provides the use of a vector or vectors containing suppression effectors in the form of nucleic acids, said nucleic acids being directed towards untranslated regions or control sequences of the target gene and vector(s) containing genomic DNA or cDNA encoding a replacement gene sequence to which nucleic acids for suppression are unable to bind, in the preparation of a combined medicament for the treatment of an autosomal dominant disease. Nucleic acids for suppression or replacement gene nucleic acids may be provided in the same vector or in separate vectors. Nucleic acids for suppression or replacement gene nucleic acids may be provided as a combination of nucleic acids alone or in vectors. The vector may contain antisense nucleic acid with or without, ribozymes.

The invention further provides a method of treatment for a disease caused by an endogenous mutant gene, said method comprising sequential or concomitant introduction of (a) antisense nucleic acids to the

non-coding regions of a gene to be suppressed; to the 5′ and/or 3′ untranslated regions of a gene or intronic regions or to the non-control regions of a gene to be suppressed, (b) replacement gene sequence with control sequences which allow it to be expressed.

The nucleic acid for gene suppression can be administered before or after or at the same time as the replacement gene is administered.

The invention further provides a kit for use in the treatment of a disease caused by an endogenous mutation in a gene, the kit comprising nucleic acids for suppression able to bind to the 5′ and / or 3′ untranslated regions or intronic regions or control regions of the gene to be suppressed and (preferably packaged separately thereto) a replacement nucleic acid to replace the mutant gene having a control sequence to allow it to be expressed.

Nucleotides can be administered as naked DNA or RNA, with or without ribozymes and/or with dendrimers. Ribozymes stabilise DNA and block transcription. Dendrimers (for example dendrimers of methylmethacrylate) can be utilised, it is believed the ~~dendrimes~~ dendrimers mimic histones and as such are capable of transporting nucleic acids into cells. Oligonucleotides can be synthesized, purified and modified with phosphorothioate linkages and 2′0-allyl groups to render them resistant to cellular nucleases while still supporting RNase H medicated degradation of RNA. Also, ~~nucelic~~ nucleic acids can be mixed with lipids to increase efficiency of delivery to somatic tissues.

Nucleotides can be delivered in vectors. Naked nucleic acids or nucleic acids in vectors can be deliv red with

lipids or other derivatives which aid gene delivery. Nucleotides may be modified to render them more stable, for example, resistant to cellular nucleases while still supporting RNaseH mediated degradation of RNA or with increased binding efficiencies as discussed earlier.

Suppression effectors and replacement sequences can be injected sub-sectionally, or may be administered systemically.

There is now an armament with which to obtain gene suppression. This, in conjunction with a better understanding of the molecular etiology of disease, results in an ever increasing number of disease targets for therapies based on suppression. In many cases, complete (100%) suppression of gene expression has been difficult to achieve. Possibly a combined approach using a number of suppression effectors may be required. For some disorders it may be necessary to block expression of a disease allele completely to prevent disease symptoms whereas for others low levels of mutant protein may be tolerated. In parallel with an increased knowledge of the molecular defects causing disease has been the realisation that many disorders are genetically heterogeneous. Examples in which multiple genes and/or multiple mutations within a gene can give rise to a similar disease phenotype include osteogenesis imperfecta, familial hypercholesteraemia, retinitis pigmentosa, and many others.

The invention addresses some shortcomings of the prior art and aims to provide a novel approach to the design of suppression effectors directed to target mutant genes. Suppression of every mutation giving rise to a disease phenotype may be costly, problematic and

sometimes impossible. Disease mutuations are often single nucleotide changes. As a result differentiating between the disease and normal alleles may be difficult. Furthermore some suppression effectors require specific sequence targets, for example, ribozymes can only cleave at NUX sites and hence will not be able to target some mutations. Notably, the wide spectrum of mutations observed in many diseases adds an additional layer of complexity in the development of therapeutic strategies for such disorders. A further problem associated with suppression is the high level of homology present in coding sequences between members of some gene families. This can limit the range of target sites for suppression which will enable specific suppression of a single member of such a gene family.

The strategy described herein has applications for alleviating autosomal dominant diseases. Complete silencing of a disease allele may be difficult to achieve using antisense, ribozyme and triple helix approaches or any combination of these. However small quantities of mutant product may be tolerated in some autosomal dominant disorders. In others a significant reduction in the proportion of mutant to normal product may result in an amelioration of disease symptoms. Hence this strategy may be applied to any autosomal dominantly inherited disease in man where the molecular basis of the disease has been established. This strategy will enable the same therapy to be used to treat a wide range of different disease mutations within the same gene. The development of strategies will be important to future gene therapies for some autosomal dominant diseases, the key to a general strategy being that it circumvents the need for a specific therapy for every dominant mutation in a given disease-causing gene. This is particularly relevant in

some disorders, for example, rhodopsin linked autosomal dominant RP (adRP), in which to date about 100 different mutations in the rhodopsin gene have been observed in adRP patients. The costs of developing designer therapies for each individual mutation which may be present in some cases in a single patient are prohibitive at present. Hence strategies such as this using a more universally applicable approach for therapy will be required.

This strategy may be applied in gene therapy approaches for biologically important polygenic disorders affecting large proportions of the world's populations such as age related macular degeneration (ARMD), glaucoma, manic depression, cancers having a familial component and indeed many others. Polygenic diseases require the inheritance of more than one mutation (component) to give rise to the disease phenotype. Notably an amelioration in disease symptoms may require reduction in the presence of only one of these components, that is, suppression of one of the genotypes which, together with others, leads to the disease phenotype, may be sufficient to prevent or ameliorate symptoms of the disease. In some cases the suppression of more than one component giving rise to the disease pathology may be required to obtain an amelioration in disease symptoms. The strategy described here may be applied broadly to possible future interventive therapies in common polygenic diseases to suppress a particular genotype(s) and thereby suppress the disease phenotype.

In the present invention suppression effectors are designed specifically to target the non-coding regions of genes, for example, the 5′ and 3′ UTRs. This provides sequence specificity for gene suppression. In

addition it provides greater flexibility in the choice of target sequence for suppression in contrast to suppression strategies directed towards single disease mutations. Furthermore it allows suppression effectors to target non-coding sequences 5′ or 3′ of the coding region thereby allowing the possibility of including the ATG start site in the target site for suppression and hence presenting an opportunity for suppression at the level of translation or inducing instability in RNA by, for example, cleavage of the RNA before the polyA tail. Notably the invention has the advantage that the same suppression strategy when directed to the 5′ and 3′ non-coding sequences could be used to suppress, in principle, any mutation in a given gene. This is particularly relevant when large numbers of mutations within a single gene cause a disease pathology. Suppression targeted to non-coding sequences allows, when necessary, the introduction of a replacement gene(s) with the same or similar coding sequences to provide the normal gene product. The replacement gene can be designed to have altered non-coding sequences and hence can escape suppression as it does not contain the target site(s) for suppression. The same replacement gene could in principle be used in conjunction with the suppression of any disease mutation in a given gene. In the case of suppression of an individual member of a gene family, the non-coding regions typically show lower levels of homology between family members thereby providing more flexibility and specificity in the choice of target sites for suppression. In relation to this aspect of the invention, the use of intronic sequences for suppression of an individual member of a family of genes has been described in a previous invention (REF: WO 92/07071). However the use of 5′ and 3′ non-coding sequences as targets for suppression holds the

advantage that these sequences are present not only in precursor messenger RNAs but also in mature messenger RNAs, thereby enabling suppressors to target all forms of RNA. In contrast, intronic sequences are spliced out of mature RNAs.

In summary the invention can involve gene suppression and replacement such that the replacement gene cannot be suppressed. Both the same suppression and replacement steps can be used for many and in some cases all of the disease mutations identified in a given gene. Therefore the invention enables the same approach to be used to suppress a wide range of mutations within the same gene. Suppression and replacement can be undertaken in conjunction with each other or separately.

Examples

The present invention is exemplified using four different genes: human rhodopsin, human peripherin, mouse rhodopsin and mouse peripherin. While all four genes are retinal specific there is no reason why the present invention could not be deployed in the suppression of other genes. Notably the 5'UTR and part of the coding sequence of the ~~COL142~~ COL1A2 gene has been cloned together with a ribozyme to target the 5'UTR of the gene ~~emphasising~~ emphasizing the broad utility of the invention in gene suppression. The 5'UTR and part of the coding sequence of the ~~COL142~~ COL1A2 gene in which there are many mutations have previously been identified which give rise to autosomal dominant osteogenesis imperfecta, has begun but was not completed at the time of submission. Many examples of mutant genes which give rise to disease phenotypes are available from the prior art - these all represent disease targets for this invention.

The present invention is exemplified using ribozymes with antisense arms to elicit RNA cleavage. There is no reason why other suppression effectors directed towards the non-coding regions of genes could not be used to achieve gene suppression. Many examples from the prior art detailing the use of suppression effectors inter alia antisense RNA/DNA, triple helix, PNAs, peptides to achieve suppression of gene expression are reported as discussed earlier. The present invention is exemplified using ribozymes with antisense arms to elicit cleavage of template RNA transcribed from one vector and non-cleavage of replacement RNAs with altered untranslated region sequences transcribed from a second vector. There is no reason why both the suppression and replacement steps could not be in the same vector. In addition there is no reason why ribozymes could not be used to combine both the suppression and replacement steps, that is, to cleave the target RNA and to ligate to the cleavage product, a replacement RNA with an altered sequence, to prevent subsequent cleavage by ribozymes which are frequently autocatalytic as discussed. The present invention is exemplified using suppression effectors directed to target the 5′ untranslated region of the above named genes. There is no reason why other non-coding regions of a gene inter alia the 3′ untranslated region or the regions involved in the control of gene expression such as promoter regions or any combination of non-coding regions could not be used to achieve gene suppression. Suppression targeted to any non-coding region of a gene would allow the expression of a replacement gene with altered sequences in the non-coding region of the gene to which the suppression effector(s) was targeted.

MATERIALS AND METHODS

Cloning vectors

cDNA templates, cDNA hybrids with altered non-coding sequences, ribozymes and antisense DNA fragments were cloned into commercial expression vectors (pCDNA3, pCDNA3, pZeoSV or pBluescript) which enable expression in a test tube from T7, T3 or SP6 promoters or expression in cells from CMV or SV40 promoters. Inserts were placed into the multiple cloning site (MCS) of these vectors typically at or near the terminal ends of the MCS to delete most of the MCS and thereby prevent any possible problems with efficiency of expression subsequent to cloning.

Sequencing protocols

Clones containing template cDNAs, hybrid cDNAs with altered non-coding sequences, ribozymes and antisense were sequenced by ABI automated sequencing machinery using standard protocols.

Expression of RNAs

RNA was obtained from clones in vitro using a commercially available Ribomax expression system (Promega) and standard protocols. RNA purifications were undertaken using the Bio-101 RNA purification kit or a solution of 0.3M sodium acetate and 0.2% SDS. Cleavage reactions were performed using standard protocols with varying $MgCl_2$ concentrations (0-15mM) at 37°C typically for 3 hours. Time points were performed at the predetermined optimal $MgCl_2$ concentrations for up to 5 hours. Radioactively labeled RNA products were obtained by incorporating α-P^{32} rUTP (Amersham) in the expression reactions (Gaughan et al. 1995). Labeled RNA products were run on polyacrylamide gels before

cleavage reactions were undertaken for the purposes of RNA purification and subsequent to cleavage reactions to establish if RNA cleavage had been achieved.

The exact base at which transcription starts has not been defined fully for some promoters (pcDNA3 Invitrogen) hence the sizes of the RNA products may vary slightly from those predicted in Table 1. In addition ~~mutiple~~ multiple rounds of cloning of a cDNA results is inserts carrying extra portions of MCS again, sometimes altering marginally the size of expressed RNA products. Typically 4-8% polyacrylamide gels were run to resolve RNA products.

RNA secondary structures

Predictions of the secondary structures of human rhodopsin, mouse rhodopsin, human peripherin, mouse peripherin and human type I Collagen COLIA2 mRNAs where obtained using the RNAPlotFold program. Ribozyme and antisense was designed to target areas of the RNA that were predicted to be accessible to suppression effectors and which were composed of non-coding sequence. The integrity of open loop structures was evaluated from the 15 most probable RNA structures. Additionally RNA structures for truncated RNA products were generated and the ~~intergrity~~ integrity of open loops between full length and truncated RNAs compared.

TEMPLATE/HYBRID/RIBOZYME AND ANTISENSE CONSTRUCTS

Examples

Various products of the examples are illustrated in Figures 1 to 20 and are explained in the results sections.

Sequences

In each case the most relevant sequences have been underlined. The position of the ATG start in each sequence is highlighted by an arrow. Sequences 1 to 18 below are represented in Figures 21 to 39 respectively. (SEQ ID NO: 1-18, respectively)

Sequence 1: Mouse ~~Rhodopsin~~ Rhodopsin cDNA sequences ~~mous~~ mouse rhodopsin 5'UTR sequences/the ATG start site/mouse rhodopsin coding sequences are shown.

Sequence 2: (F+R) Mouse Rhodopsin cDNA with altered non-coding sequences mouse rhodopsin 5'UTR sequences with a 1 base change/the ATG start site/mouse rhodopsin coding sequences are shown.

Sequence 3: (F+R) Mouse Rhodopsin cDNA with altered non-coding sequences mouse rhodopsin 5'UTR sequences with a 1 base change/the ATG start site/mouse rhodopsin coding sequences are shown.

Sequence 4: Ribozyme 3

Sequence 5: Human Rhodopsin cDNA sequence human rhodopsin 5'UTR sequences/the ATG start site/human rhodopsin coding sequences are shown.

Sequence 6: Human Rhodopsin cDNA with altered non-coding sequences human rhodopsin 5'UTR sequences (shorter UTR)/the ATG start site/human rhodopsin coding sequences are shown.

Sequence 7: Ribozyme 15

Sequence 8: Mouse ~~perhiperin~~ peripherin cDNA sequences mouse peripherin 5'UTR sequences/the ATG start site/mouse peripherin coding sequences are shown.

Sequence 9: Mouse ~~perhiperin~~ peripherin cDNA with altered non-coding sequences mouse rhodopsin 5'UTR sequences/the ATG start site/mouse peripherin coding sequences are shown.

Sequence 10: Ribozyme 17

Sequence 11: Human peripherin cDNA sequences human peripherin 5'UTR sequences/the ATG start site/human peripherin coding sequences are shown.

Sequence 12: Human peripherin cDNA with altered non-coding sequences Partial human and mouse peripherin 5'UTR sequences/the ATG start site/human peripherin coding sequences are shown.

Sequence 13: Ribozyme 8

Sequence 14: Ribozyme 9

Sequence 15: Human type I collagen (~~col1A2~~ col1A2) sequence - 5'UTR and exon 1 sequence

Sequence 16: Ribozyme 18

Sequence 17: Antisense construct containing 127bp of antisense ~~sequence~~ SEQUENCE targeting the 5'UTR of the mouse peripherin gene.

Sequence 18: Sense construct containing 127bp of sense sequence from the 5'UTR of the mouse peripherin gene.

Mouse Rhodopsin

Template cDNA

A full length mouse rhodopsin cDNA was generated from a partial cDNA clone missing the sequence coding for the first 20 amino acids of the protein and a partial genomic clone which enabled the production of a full length cDNA (kindly donated by Dr Wolfgang Baehr). The full length cDNA was cloned into the EcoRI site of [pCDNA3] pcDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoters in the vector. The full length 5'UTR sequence was present in this clone. In addition to the full length 5' UTR sequence the clone contains additional 5' upstream sequence of the mouse rhodopsin gene as the clone was generated using the EcoRI site present at position 1120 (Accession number: M55171). (~~Sequence~~ SEQ ID NO: 1)

Hybrid cDNAs with altered non-coding regions

Hybrid I

The mouse rhodopsin hybrid cDNA sequence was altered in the non-coding sequences by PCR primer directed mutagenesis and cloned into the HindIII and EcoRI sites of [pCDNA3] pcDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoters in the vector. PCR mutagenesis was undertaken using a HindIII (in the MCS of pCDNA3) to Eco47III (in Exon 2 of the

gene) DNA fragment. The 5'UTR was altered significantly - the mouse rhodopsin 5'UTR was completely replaced by the 5'UTR of the human peripherin gene, that is, by 5'UTR sequence from a different gene (peripherin) and from a different species (human) but from a gene expressed in the same tissue as mouse rhodopsin, i.e., photoreceptor cells (~~Sequence~~ SEQ ID NO: 2). The sequence of the mouse rhodopsin cDNA is present in the clone from the ATG start onwards.

Hybrid 2

The mouse rhodopsin hybrid cDNA sequence was altered in the non-coding sequences to eliminate the GUC ribozyme binding site targeted in the 5'UTR of mouse rhodopsin. The U of the target was changed to G, that is, GUC-->GGC (~~Sequence~~ nucleotides 340-342 of SEQ ID NO: 3). Again PCR mutagenesis was primer driven and was undertaken using a ~~HindIII~~ HindIII (in pCDNA3) to Eco47111 (in the coding sequence of the mouse rhodopsin cDNA) DNA fragment.

Ribozyme constructs

A hammerhead ribozyme (termed Rib3) designed to target an open loop structure in the RNA in the 5' non-coding region of the gene was cloned into the ~~HindIII~~ HindIII and ~~XbaI~~ XbaI sites of pCDNA3 again allowing subsequent expression of RNA from the T7 or CMV promoters in the vector (~~Sequence~~ SEQ ID NO: 4). The target site was GUC at ~~position~~ position 1393-1395 of the mouse rhodopsin sequence (Accession number: M55171). Antisense flanks are underlined.

Rib3: CUUCGUACUGAUGAGUCCGUGAGGACGAAACAGAGAC (nucleotides 95-131 of SEQ ID NO: 4)

Human Rhodopsin

Template cDNA

The human rhodopsin cDNA was cloned into the ~~HindIII~~ HindIII and EcoRI sites of the MCS of pCDNA3 in a 5' to 3'

orientation allowing subsequent expression of RNA from the T7 or CMV promoters in the vector. The full length 5'UTR sequence was inserted into this clone using primer driven PCR mutagenesis and a ~~HindIII~~ (in pCDNA3) to BstEII (in the coding sequence of the human rhodopsin cDNA) DNA fragment (~~Sequence~~ 5)

Hybrid cDNAs with altered non-coding regions

The human rhodopsin hybrid cDNA with ~~alterrd~~ non-coding sequences was cloned into the EcoRI site of pCDNA3 in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoters in the vector. The 5'UTR of this clone included only the first 21 bases of the non-coding region of human rhodopsin before the ATG start site (~~Sequence~~ 6).

Ribozyme constructs

A hammerhead ribozyme (termed Ribl5) designed to target an open loop structure in the RNA from the non-coding regions of the gene was cloned subsequent to synthesis and annealing into the ~~HindIII~~ and ~~XbaI~~ sites of pCDNA3 again allowing subsequent expression of RNA from the T7 or CMV promoters in the vector (~~Sequence~~ 7). The target site was AUU (the NUX rule) at position 249-251 of the human rhodopsin sequence (Accession number: K02281). Antisense flanks are underlined. Ribl5:

ACCCAAGCUGAUGAGUCCGUGAGGACGAAAUGCUGC

Mouse Peripherin

Template cDNA

A mouse peripherin cDNA was cloned into the ~~HindIII~~ and EcoRV sites of pCDNA3. The clone is in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 or CMV promoters in the vector (~~Sequence~~ 8). The clone contains the compl te 5'UTR sequence together

with 27 bases of additional sequence 5′ of this UTR sequence left probably from other cloning vectors.

Hybrid cDNAs with altered non-coding regions

The mouse peripherin hybrid cDNA was altered in the 5′non-coding region. Using primer driven PCR mutagenesis the mouse rhodopsin 5′UTR sequence was replaced by the sequence of the mouse peripherin 5′UTR (~~Sequence~~ SEQ ID NO: 9). The PCR mutagenesis was achieved using a ~~HindIII~~ HindIII (in ~~pCDNA3~~ pcDNA3) to ~~SacII~~ SacII (in the coding sequence of the mouse peripherin cDNA) DNA fragment.

Ribozyme constructs

A hammerhead ribozyme (termed Rib17) designed to target an open loop structure in the RNA from the non-coding regions of the gene was cloned into the ~~Hind III~~ HindIII and ~~XbaI~~ XbaI sites of ~~pCDNA3~~ pcDNA3 again allowing subsequent expression of RNA from the T7 or CMV promoters in the vector (~~Sequence~~ SEQ ID NO: 10). The target site was AUU at position 162-164 of the mouse peripherin sequence (Accession number: X14770). Antisense flanks are underlined. Rib17:

CACUCCUCUGAUGAGUCCGUGAGGACGAAAUCCGAGU (nucleotides 99-136 of SEQ ID NO: 10)

Antisense constructs

Antisense and sense constructs were PCR amplified and cloned into ~~pCDNA3~~ pcDNA3 and pZEOSV for expression *in vitro* and *in vivo*. For example, a 127bp fragment from the 5′UTR sequence of mouse peripherin was cloned in both orientations into the above stated vectors. The effectiveness of antisense at suppression is under evaluation. The altered hybrid cDNA clones are being used to establish if RNAs expressed from these altered clones are protected from antisense suppression effects (~~Sequences~~ SEQ ID NOS: 17 and 18).

Human Peripherin

Template cDNA

A human peripherin cDNA cloned into the EcoRI site of the commercially available vector pBluescript was kindly provided by Dr. Gabriel Travis. The clone is in a 5′ to 3′ orientation allowing subsequent expression of RNA from the T7 promoter in the vector. The full length 5′UTR sequence is present in this clone (~~Sequence~~ SEQ ID NO: 11).

Hybrid cDNAs with altered non-coding regions

The hybrid clone with altered non-coding sequences was generated as follows. The hybrid clone contains human RDS 5′UTR sequences until the BamHI site in the human peripherin 5′UTR sequence. From this site the clone runs into mouse RDS 5′UTR sequence until the ATG start site where it returns to human RDS sequence (~~Sequence~~ SEQ ID NO: 12). The clone was generated using primer driven PCR mutagenesis of a BamHI (in the 5′UTR sequence) to Bgl1 (in the coding sequence of the human peripherin cDNA) DNA fragment.

Ribozyme constructs

Hammerhead ribozymes (termed Rib8 and Rib9) designed to target open loop structures in the RNA from the non-coding regions of the gene were cloned into the ~~HindIII~~ HindIII and ~~XbaI~~ XbaI sites of pcDNA3 pCDNA 3 which again allows subsequent expression of RNA from the T7 or CMV promoters in the vector (~~Sequences~~ SEQ ID NOS: 13 and 14). The target sites were CUA and GUU at positions 234-236 and 190-192 respectively of the human peripherin sequence (Accession number: M62958). Rib8: CCAAGUGCUGAUGAGUCCGUGAGGACGAAAGUCCGG (nucleotides 93-128 of SEQ ID NO: 13)

Rib9: CAAACCUUCUGAUGAGUCCGUGAGGACGAAACGAGCC Antisense

flanks are underlined. (nucleotides 94-130 of SEQ ID NO: 14)

Human Type I C llagen - COL1A2

Template cDNA

A partial human type I collagen 1A2 cDNA sequence including the 5'UTR sequence and exon 1 was cloned after PCR amplification into the ~~HindIII~~ and ~~XhoI~~ sites of [pCDNA3]. The clone is in a 5' to 3' orientation allowing subsequent expression of RNA from the T7 and or CMV promoters in the vector (~~Sequence~~ 15). The clone contains the complete 5'UTR sequence together with Exon I of COL1A2.

Ribozyme ~~construct~~

A hammerhead ribozyme (termed Rib18) designed to target an open loop structure in the RNA from the non-coding regions of the gene was cloned into the ~~HindIII~~ and ~~XbaI~~ sites of [pCDNA3] again allowing subsequent expression of RNA from the T7 or CMV promoters in the ~~vfftor~~ (Sequence 16). The target site was GUC at position 448-450 of the human type I collagen 1A2 sequence (Accession number: J03464; M18057; X02488). Antisense flanks are underlined. Rib18:

AGACAUGCCUGAUGAGUCCGUGAGGACGAAACUCCUU

RESULTS

Human and mouse rhodopsin and peripherin cDNAs were expressed *in vitro*. Likewise human and mouse rhodopsin and peripherin cDNAs with altered 5'non-coding sequences were expressed *in vitro*. Ribozymes targeting the 5'UTRs of these retinal cDNAs were also expressed *in vitro*. cDNA clones were cut with various restriction enzymes resulting in the production of differently sized RNAs after expression. This aided in differentiating between RNAs expressed from the original cDNAs or from altered hybrid cDNAs. The sites used to cut each clone, the predicted sizes of the resulting RNAs and the predicted sizes of cleavage

products after cleavage by target ribozymes are given below in Table 1.

TABLE 1

	Restriction Enzyme	RNA Size	Cleavage Products
Example 1 Mouse rhodopsin Mouse rhodopsin hybrid 1 Mouse rhodopsin hybrid 2 Rib 3 (See Table 1; sequences 1-4; Figures 1-6; Figures 1-6)	 Eco47111 Eco47111 Fsp 1 Xho 1	 778 bases 643 bases 577 bases 60 bases	 336 + 442 bases
Example 2 Human rhodopsin Human rhodopsin hybrid Rib 15 (See Table 1; sequences 5-7; figures 7-11)	 BstEII Acy 1 BstEII Acy 1 Fspl Xba1	 8511 bases 1183 bases 841 bases 1173 bases 300 bases 55 bases	 61 + 790 bases 61 + 1122 bases
Example 3 Mouse peripherin Mouse peripherin hybrid Rib 17 (See Table 1; sequences 8-10; figures 12-15)	 Bgl1 Bgl1 Xba1	 488 bases 344 bases 60 bases	 201 + 287 bases
Example 4 Human peripherin Human peripherin hybrid Rib 8 Rib 9 (see Table 1; sequence 11-14; figures 16-19)	 Bgl1 Avrll Xbal Xbal	 489 bases 331 bases 55 bases 55 bases	 238 + 251 (Rib 8) 194 + 295 (Rib 9)
Example 5 Collagen 1A2 Rib 18 (See Table 1; sequences 15 and 16)	 Xhol Xbal		
Example 6 Antisense constructs (See Table 1; sequences 17 and 18)			

The examples of the invention are illustrated in the

accompanying figures wherein:

Figure 1 pBR322 was cut with MspI, radioactively labeled and run on a polyacrylamide gel to enable separation of the resulting DNA fragments. The sizes of these fragments are given in Figure 1. This DNA ladder was then used on subsequent polyacrylamide gels to provide an estimate of the size of the RNA products run on the gels.

Figure 2

A: Mouse rhodopsin cDNA was expressed from the T7 promoter to the Eco47III site in the coding sequence. The RNA was mixed with Rib3 RNA with varying concentrations of magnesium chloride. Lane 1-4: Rhodopsin RNA and Rib3 RNA after incubation for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Complete cleavage of mouse rhodopsin RNA was obtained with a small residual amount of intact RNA present at 5mM magnesium chloride. Note at 0mM magnesium chloride before activation of Rib3 RNA no cleavage products were observed.

B: Mouse rhodopsin cDNA was expressed from the T7 promoter to the Eco47III site in the coding sequence. Resulting RNA was mixed with Rib3 RNA with varying concentrations of magnesium chloride. Lane 1: DNA ladder as in Figure 1. Lane 2: intact mouse rhodopsin RNA. Lane 3-6: Rhodopsin RNA and Rib3 RNA after incubation for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. Again complete cleavage of mouse rhodopsin RNA was obtained with a small residual amount of intact RNA present at 5mM magnesium chloride. Lane 7: DNA ladder as in Figure 1.

Figure 3

Mouse rhodopsin cDNA was expressed from the T7 promoter to the Eco47III site in the coding sequence. Lane 1: intact mouse rhodopsin RNA. Lanes 2-7: Mouse rhodopsin RNA was mixed with Rib3 RNA with 15mM magnesium chloride and incubated at 37°C for 0, 30, 60, 90, 120 and 180 minutes. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Complete cleavage of mouse rhodopsin RNA was obtained. Notably cleavage was observed immediately after the addition of the divalent ions which activated Rib3 RNA (see Lane 2: 0 minutes).

Figure 4

Mouse rhodopsin cDNA with altered 5'UTR sequence was expressed from the T7 promoter to the Eco47III site in the coding sequence. The resulting RNA was mixed with Rib3 RNA using varying concentrations of magnesium chloride. Lane 1: DNA ladder as in ~~Diagram~~ Figure 2. Lane 2: intact altered mouse rhodopsin RNA. Lane 3 6: altered mouse rhodopsin RNA and Rib3 RNA after incubation for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. No cleavage of the altered hybrid RNA ~~was~~ occurred.

Figure 5

Mouse rhodopsin cDNA with altered 5'UTR sequence was expressed from the T7 promoter to the Eco47III site in the coding sequence. The resulting RNA was mixed with Rib3 RNA with 10mM magnesium chloride and incubated at 37°C. Lane 1: intact altered mouse rhodopsin RNAs. Lane 2-6: altered mouse rhodopsin RNA and Rib3 RNA after incubation for 0, 30, 60,120, 180 minutes. No cleavage of the hybrid RNA was obtained. Notably after 3 hours incubation with Rib3 RNA the adapted mouse rhodopsin RNA was as intense as at 0 minutes. Lane 7: DNA ladder as

in Figure 1.

Figure 6

A: The unadapted mouse rhodopsin cDNA and the mouse rhodopsin cDNA with altered 5'UTR sequence were expressed from the T7 promoter to the Eco47III site in the coding sequence. The resulting RNAs were mixed together with Rib3 RNA and 10mM magnesium chloride. Lane 1: intact unadapted and altered mouse rhodopsin RNAs which can clearly be differentiated by size as predicted (Table 1). Lane 2-6: unadapted and altered mouse rhodopsin RNAs and Rib3 RNA after incubation for 0, 30, 60,120,180 minutes with 10mM magnesium chloride at 37°C. No cleavage of the altered hybrid RNA was obtained. The hybrid was of equal intensity after 3 hours as it was at 0 minutes. Notably the majority of the unadapted mouse rhodopsin RNA is cleaved immediately by Rib3 RNA even in the presence of the altered mouse rhodopsin RNA. The cleavage products are highlighted with arrows. The background is due to a small amount of RNA degradation. B: In a separate experiment the three RNAs (unadapted, altered mouse rhodopsin RNAs and Rib3 RNA) were incubated at 15mM magnesium chloride for 5 hours. The altered hybrid RNA remains intact but the unadapted mouse rhodopsin RNA has been cleaved completely.

Figure 7

A second altered mouse rhodopsin cDNA involving a single base change at the ribozyme cleavage site was generated. This adapted mouse rhodopsin cDNA was expressed from the T7 promoter to the FspI site in the coding sequence. Likewise the unadapted mouse rhodopsin cDNA was expressed from the T7 promoter to the Eco47III site in the coding sequence. These two RNAs were mixed with Rib3 RNA and incubated at 37°C with 15mM magnesium

chloride. Lane 1: Intact mouse rhodopsin RNA. Lane 2: Intact altered mouse rhodopsin RNA (2nd alteration). Lane 3: DNA ladder as in ~~Diagram~~ Figure 1. Lanes 4-7: Unadapted and altered mouse rhodopsin RNAs and Rib3 RNA after incubation for 0, 60, 120 and 180 minutes ~~wim~~ with 15mM magnesium chloride at 37°C. Note the reduction of the unadapted RNA product over time in the presence of the altered RNA (Lanes 4 and 5). The adapted RNA remains intact and maintains equal ~~in~~ intensity at each time point indicating that it is resistant to cleavage by Rib3 RNA. Again, as with all other altered RNAs, no additional cleavage products were observed. Lane 8: The unadapted and adapted mouse rhodopsin RNA without ribozyme. Lane 9: DNA ladder as in ~~Diagram~~ Figure 1.

Figure 8

Human rhodopsin was expressed from the T7 promoter to the BstEII site in Exon IV. The resulting RNA was mixed with Rib15 RNA with varying concentrations of magnesium chloride. Lane 1: intact rhodopsin RNA alone. Lane 2: Rib15 alone. Lane 3: DNA ladder as in ~~Diagram~~ Figure 1. Lanes 4-7: Rhodopsin RNA and Rib15 RNA after incubation for 3 hours at 37°C with the 0mM, 5mM, 10mM and 15mM magnesium chloride. Predicted cleavage products are 61 and 790 bases (Table 1). Lane 8: DNA ladder. Partial cleavage of the RNA was obtained - a doublet representing the intact RNA and the larger cleavage product is present (most clearly in lane 5). The gel was run a shorter distance than the gel presented in Figure ~~8-11~~ 9-12 to show the presence of Rib15 RNA at the bottom of the gel and to demonstrate that one of the cleavage products cannot be ~~visualised~~ visualized due to the presence of the labeled ribozyme which runs at approximately the same size. Subsequent gels were run further to achieve better separation of these two RNA fragments.

Figure 9

Both the unadapted human rhodopsin cDNA and the altered cDNA were expressed from the T7 promoter to the BstEII site in Exon IV. The resulting RNA was mixed with Rib15RNA with varying concentrations of magnesium chloride. Lane 1: intact human rhodopsin RNA alone. Lane 2: DNA ladder as in Figure 1. Lane 3-6: Rhodopsin RNA and Rib15RNA after incubation together for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. Lane 7: DNA ladder as in Figure 1. Lane 8-11: Human rhodopsin RNA with altered 5'UTR sequence and Rib15RNA after incubation together for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. Lane 12: intact human rhodopsin RNA with altered 5'UTR sequence alone. The predicted cleavage products for human rhodopsin are 61 and 790 bases (Table 1) - the larger cleavage product is clearly visible when the ribozyme becomes active after the addition of magnesium chloride (Lanes 4-6). This larger cleavage product is highlighted by an arrow.

Figure 10

Human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in Exon IV. Likewise the altered human rhodopsin cDNA was expressed from the T7 promoter to the FspI site in Exon 1. Both resulting RNAs were mixed together with Rib15RNA with varying concentrations of magnesium chloride. Lane 1: DNA ladder as in Figure 1. Lanes 2-5: Rhodopsin RNA, altered rhodopsin RNA and Rib15RNA after incubation for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Partial cleavage of the unadapted RNA was obtained after magnesium was added to the reaction. The altered human rhodopsin RNA was protected from cleavage in all reactions. If cleavage of the altered human

rhodopsin RNA had ~~occoured~~ occurred the products rationally would most likely be of a different size than those observed with the unadapted RNA. Notably no additional cleavage products were observed. Moreover there was no change in intensity of the altered RNA when the ribozyme was active (in the presence of magnesium chloride) or inactive (at 0mM magnesium chloride). In contrast the undapted human rhodopsin RNA is less intense in lanes 3-5 after cleavage than in lane 2 before the addition of magnesium to activate Rib15. Lane 6: intact human rhodopsin RNA. Lane 7: intact human rhodopsin RNA with altered 5'UTR sequence. Lane 8: DNA ladder.

Figure ~~10~~ 11

Human rhodopsin cDNA was expressed from the T7 promoter to the BstEII site in Exon IV. Likewise the altered human rhodopsin cDNA was expressed from the T7 promoter to the Acyl in the 3'rhodopsin sequence after the stop codon. Both resulting RNAs were mixed together with Rib15/RNA with varying concentrations of magnesium chloride. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lanes 2-5: Rhodopsin RNA, altered rhodopsin RNA and Rib15/RNA after incubation for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. Lane 6: Intact human rhodopsin RNA. Lane 7: DNA ladder as in ~~Diagram~~ Figure 1. Note that neither RNAs or cleavage products are present in Lane 5 as too little sample may have been loaded in this lane.

Figure ~~11~~ 12

Human rhodopsin cDNA and the cDNA with altered 5'sequence were expressed from the T7 promoter to the Acyl site after the coding sequence of human rhodopsin. The resulting RNA was mixed ~~wim~~ with Rib15/RNA with varying concentrations of magnesium chloride. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2-5: Human rhodopsin RNA

and Rib15RNA after incubation together for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. Lane 6: Intact human rhodopsin RNA. Lane 7: DNA ladder as in ~~Diagram~~ Figure 1. Lane 8-11: Human rhodopsin RNA with altered 5'UTR sequence and Rib15RNA after incubation together for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. Lane 12: intact human rhodopsin RNA with altered 5'UTR sequence alone. Lane 13: DNA ladder as in ~~Diagram~~ Figure 1. The larger of the predicted cleavage products is present in lanes 3-5 and is highlighted by an arrow. The adapted human rhodopsin RNA again was protected from cleavage by Rib15^{V}RNA. Note that in Lane 12 too little sample may have been loaded.

Figure ~~12~~ 13
Mouse peripherin cDNA was expressed from the T7 promoter to the BgIII site in the coding sequence. The RNA was mixed with Rib17RNA with varying concentrations of magnesium chloride. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2: intact mouse peripherin RNA. Lanes 3-6: Mouse peripherin RNA and Rib17RNA after incubation for 3 hours at 37°C ~~with~~ with 0mM, 5mM, 10mM and 15mM magnesium chloride. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Partial cleavage of mouse rhodopsin RNA was obtained once Rib17^{V}RNA was activated with magnesium chloride. Possibly some of the RNA was in a conformation that was inaccessible to Rib17^{V}RNA. It should be noted that in the ~~absense~~ absence of magnesium chloride the ribozyme was inactive and no cleavage products were observed.

Figure ~~13~~ 14
Mouse peripherin cDNA was expressed from the T7 promoter to the ~~BgIII~~ BglII site in the coding sequence. The resulting RNA was mixed with Rib17RNA with 15mM magnesium chloride and incubated at 37°C for varying

times. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2: intact mouse peripherin RNA. Lanes 3-6: Mouse peripherin RNA and Rib17 RNA after incubation together with 15mM magnesium chloride at 37°C for 0,1, 2 and 3 hours respectively. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Partial cleavage of mouse rhodopsin RNA was obtained with Rib17 after 1 hour. The proportion of the RNA cleaved increased over time. The intensity of the mouse rhodopsin RNA band decreased visibly on the gel by 3 hours and similarly the cleavage products visibly increased in intensity. It is possible that further cleavage might be obtained over longer time periods. Lane 7: DNA ladder as in ~~Diagram~~ Figure 1.

Figure ~~14~~ 15

Mouse peripherin cDNA with altered 5'sequences was expressed from the T7 promoter to the ~~BglII~~ BglII site in the coding sequence. The resulting RNA was mixed with Rib17 RNA with varying concentrations of magnesium chloride. Lane 1: intact altered mouse peripherin RNA with no ribozyme. Lanes 2-5: Mouse peripherin RNA with altered 5'sequence and Rib17 RNA after incubation for 3 hours at 37°C with 0mM, 5mM, 10mM and 15mM magnesium chloride. The sizes of the expressed RNAs are as expected (Table 1). No cleavage of the adapted mouse rhodopsin RNA was obtained before or after Rib17 RNA was activated with magnesium chloride. Lane 6: DNA ladder as in ~~Diagram~~ Figure 1.

Figure ~~15~~ 16

Both the unadapted and adapted mouse peripherin cDNAs were expressed from the T7 promoter to the ~~BglII~~ BglII site in the coding ~~sequence~~ sequence. The resulting RNAs were mixed together with Rib17 RNA with 15mM magnesium chloride and incubated at 37°C for varying times. Lane 1: DNA ladder

as in Figure 1. Lane 2: intact unadapted and altered mouse peripherin RNA. Lanes 3-6: Unadapted mouse peripherin RNA, altered mouse peripherin RNA and Rib17 RNA after incubation together with 15mM magnesium chloride at 37°C for 0, 30, 90 and 180 minutes respectively. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Partial cleavage of the unadapted mouse peripherin RNA was obtained with Rib17 RNA after 1 hour. The intensity of the larger unadapted mouse peripherin RNA product decreases slightly over time. In contrast the cleavage products increase in intensity. The intensity of the smaller altered mouse peripherin RNA product remains constant over time indicating that the RNA is not cleaved by Rib17 RNA. Lane 7: DNA ladder as in Figure 1.

Figure 17

Both the unadapted and adapted human peripherin cDNAs were expressed from the T7 promoter to the Bg1II site in the coding sequence. The resulting RNAs were mixed together with Rib8 RNA with varying concentrations of magnesium chloride and incubated at 37°C for 3 hours. Lane 1: Unadapted human peripherin without ribozyme. Lanes 2-5: Unadapted human peripherin RNA and Rib8 RNA after incubation together with 0, 5, 10, 15mM magnesium chloride respectively at 37°C for 3 hours. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Almost complete cleavage of the unadapted human peripherin RNA was obtained with Rib8 RNA after 3 hours. The intensity of the larger unadapted human peripherin RNA product decreases over time. Lanes 6-9: Altered human peripherin RNA and Rib8 RNA after incubation together with 0, 5,10,15mM magnesium chloride respectively at 37°C for 3 hours. The sizes of the expressed RNAs are as expected (Table 1). No cleavage of the alter d human peripherin RNA was obtained with

Rib8 RNA even after 3 hours. The intensity of the smaller altered mouse peripherin RNA product remains constant (with the exception of lane 9 in which less sample may have been loaded) indicating that the RNA is not cleaved by Rib8 RNA. In addition no cleavage products were observed. Lane 10: Intact unadapted human peripherin RNA alone. Lane 11: Intact altered human peripherin RNA alone. Lane 12: DNA ladder as in ~~Diagram~~ Figure 1.

Figure ~~17~~ 18

The unadapted and altered human peripherin cDNAs were expressed from the T7 promoter to the Bg1II site in the coding sequence. The resulting RNAs were mixed together with Rib8 RNA for varying times with 15mM magnesium chloride and incubated at 37°C. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lane 2-5: Unadapted and altered human peripherin RNAs and Rib8 RNA after incubation together for 0,1, 2 and 3 hours respectively at 37°C with 15mM magnesium chloride. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Almost complete cleavage of the larger unadapted human peripherin RNA was ~~obtainffd~~ obtained with Rib8 RNA after 3 hours. The intensity of the larger unadapted human peripherin RNA product decreases over time. Altered human peripherin RNA was not cleaved by Rib8 RNA even after 3 hours. The intensity of the smaller altered mouse peripherin RNA product remains constant over time indicating that the RNA is not cleaved by Rib8 RNA. In addition no additional cleavage products were observed. Lane 6: Intact unadapted and altered human peripherin RNA together without ribozyme. Lane 7: DNA ladder as in ~~Diagram~~ Figure 1.

Figure ~~18~~ 19

Both the unadapted and adapted human peripherin cDNAs were expressed from the T7 promoter to the Bg1II site

in the coding sequence. The resulting RNAs were mixed together with Rib9 RNA with varying ~~concentrations~~ concentrations of magnesium chloride and incubated at 37°C for 3 hours. Lane 1: DNA ladder as in ~~Diagram~~ Figure 1. Lanes 2-5: Unadapted human peripherin RNA and Rib9 RNA after incubation together with 0, 5, 10, 15mM magnesium chloride respectively at 37°C for 3 hours. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Almost complete cleavage of the unadapted human peripherin RNA was obtained with Rib9^{v} RNA. The intensity of the larger unadapted human peripherin RNA product decreases greatly. Lanes 6-9: Altered human peripherin RNA and Rib9 RNA after incubation together with 0, 5, 10, 15mM magnesium chloride respectively at 37°C for 3 hours. The sizes of the expressed RNAs are as expected (Table 1). No cleavage of the altered human peripherin RNA was obtained with Rib17^{v} RNA even after 3 hours. The intensity of the smaller altered mouse peripherin RNA was observed - the product remains constant over time indicating that the RNA is not cleaved by Rib9$_{\Delta}$ RNA. Lane 10: Intact unadapted human peripherin RNA alone. Lane 11: Intact altered human peripherin RNA alone. Lane 12: DNA ladder as in ~~Diagram~~ Figure 1. Rib9$_{\Delta}$ RNA was ~~designed~~ designed to target a different loop structure in the 5'sequence of human peripherin. It may result in slightly more efficient cleavage of RNA than Rib8^{v} RNA.

Figure ~~19~~ 20

The unadapted and altered human peripherin cDNAs were expressed from the T7 promoter to the ~~BglII~~ BglII site in the coding sequence. The resulting RNAs were mixed together with Rib9 RNA for varying times with 15mM magnesium chloride and incubated at 37°C. Lane 1: Intact unadapted human peripherin RNA without ribozyme. ~~Iane~~ Lane 2: Intact altered human peripherin RNA without

ribozyme. Lanes 3 and 4: DNA ladder as in ~~Diagram~~ Figure 1. Lane 5-8: Unadapted and altered human peripherin RNAs and Rib9 RNA after incubation together for 0,1, 2 and 3 hours respectively at 37°C with 15mM magnesium chloride. The sizes of the expressed RNAs and cleavage products are as expected (Table 1). Cleavage products were observed at time zero. Almost complete cleavage of the larger unadapted human peripherin RNA was obtained with Rib9v RNA after 1 hour. The intensity of the larger unadapted human peripherin RNA product decreased quickly over time. The altered human peripherin RNA was not cleaved by Rib9v RNA even after 3 hours. The intensity of the smaller altered human peripherin RNA product remains constant over time indicating that the RNA is not cleaved by Rib9v RNA. In addition no additional cleavage products were observed. Lane 9: Intact unadapted and altered human peripherin RNA ~~togemer~~ together without ribozyme. Lane 10: DNA ladder as in ~~Diagram~~ Figure 1.

Example 1

Mouse Rhodopsin

Rib3v RNA targeting the mouse rhodopsin 5'non-coding sequence was cut with Xho I and expressed *in vitro*. The mouse rhodopsin cDNA and hybrid cDNA with altered 5'non-coding sequence (with the human peripherin 5'UTR sequence in place of the mouse rhodopsin 5'UTR sequence) were cut with Eco47111, expressed and both RNAs mixed separately and together with Rib3 RNA to test for cleavage. RNAs were mixed with varying concentrations of $MgCl_2$ and for varying amounts of time to optimise cleavage of RNA by Rib3v RNA (Figures ~~1-6~~ 2-7). Likewise a second hybrid with a small modification of the 5'UTR ~~sfrquence~~ sequence was cut with Fspl, expressed and tested for cleavage with Rib3 RNA alone and together

with the original unadapted mouse rhodopsin RNA. This alteration is a single base change at the ribozyme cleavage site involving a U-->G, that is, altering the ribozyme cleavage site from GUC to GGC thereby removing the target site. In all cases the expressed RNA was the correct size. In all cases cleavage of the larger unadapted mouse rhodopsin RNA product was achieved. In some cases cleavage was complete and all cleavage products were of the predicted size. Notably hybrid mouse rhodopsin RNAs with altered 5'UTR sequences were not cleaved by Rib3 RNA either when mixed alone with Rib3 RNA or when combined with Rib3 RNA and the unadapted mouse rhodopsin RNA (Figures ~~1-6~~ 2-7). This highlights the sequence specificity of the Rib3 RNA target in that small sequence alterations may be all that is required to prevent cleavage. Likewise small modifications in the targets for the antisense arms of ribozymes or more generally for any antisense may result in the inability of a suppression effector to attack the modified RNA. The first hybrid described above could be used to prevent ribozyme cleavage or antisense binding of many ribozymes or antisense suppression effectors and therefore would be particularly useful if more than one suppression effector was required to achieve suppression.

Example 2

Human Rhodopsin

The human rhodopsin cDNA clone (with a full length 5'UTR) and the human rhodopsin hybrid cDNA clone with altered 5'non-coding sequence (shorter 5'UTR) were cut with BstEII and expressed *in vitro*. The Rib15 clone was cut with XbaI and expressed *in vitro*. The resulting ribozyme and human rhodopsin RNAs were mixed with varying concentrations of $MgCl_2$ to ~~optimise~~ optimize cleavage of

the template RNA by Rib15 RNA (Figures 8-12). The human rhodopsin cDNA and hybrid cDNA with altered 5'non-coding sequence were cut with Acyl, expressed and both RNAs mixed separately (due to their similar sizes) with Rib15 RNA to test for cleavage (Figures 8-12). The human rhodopsin cDNA was cut with BstEII and the hybrid cDNA with altered 5'non-coding sequence cut with Fspl, expressed and mixed separately and together with Rib15 RNA to test for cleavage (Figures 8-12). In all cases the expressed RNA was the correct size. Similarly in all cases the unadapted RNA template was cut into cleavage products of the predicted sizes. The cleavage of the unadapted RNA template was incomplete with some residual uncleaved RNA remaining. This may be due, for example, to the inability of the ribozyme to access RNA in some conformations. In all cases RNA expressed from the altered hybrid human rhodopsin cDNA with a shorter 5'UTR remained intact, that is, it was not cleaved by Rib15 RNA. It is worth noting that Acyl enzyme cuts after the stop codon of the coding region of the gene and therefore the resulting RNA includes all of the coding sequence that gives rise to the protein. The RNA from the original unadapted human rhodopsin cDNA clone cut with Acyl is cleaved by Rib15 RNA. In contrast, RNA from the hybrid clone with an altered 5'UTR sequence is not cleaved by Rib15 RNA (Figures 8-12). The sequence of the ribozyme target site and of the antisense flanks are not present in the altered human rhodopsin RNA. Clearly, altering the sequence in non-coding regions masks the resulting altered gene from being suppressed by antisense or ribozymes targeting sites in non-coding regions.

Example 3

Mouse Peripherin

Rib17 targeting mouse peripherin 5'non-coding sequence was cut with Xbal and expressed in vitro. The mouse peripherin cDNA and mouse peripherin hybrid cDNA with an altered 5'non-coding sequence (in which the mouse peripherin 5'UTR sequence has been replaced by mouse rhodopsin 5'UTR sequence) were cut with ~~Bg111~~, BglII expressed in vitro and both RNAs mixed separately and together with Rib17 RNA to test for cleavage. RNAs were mixed with varying concentrations of $MgCl_2$ and for varying times to ~~optimise~~ optimise cleavage of RNAs by Rib17^V RNA (Figures ~~12-15~~ 13-16). Partial cleavage of the unadapted mouse peripherin RNA by Rib17^V RNA was obtained - all RNAs expressed and all cleavage products were the predicted sizes. Partial cleavage may be due to the inaccessibility of some RNA conformations to antisense binding and/or ribozyme cleavage. In contrast the adapted hybrid mouse peripherin RNA containing mouse rhodopsin non-coding sequences remained intact (Figures ~~12-15~~ 13-16). This again highlights that RNAs can be designed so that they code for a correct protein, in this case, mouse peripherin and such that they are masked from a suppression effector(s), in this case, a ribozyme with antisense flanks.

Example 4

Human Peripherin

Rib8 and Rib9 clones targeting human peripherin 5'non-coding sequence were cut with Xbal and expressed *in vitro*. The human peripherin cDNA and human peripherin hybrid cDNA with altered 5'non-coding sequence (with part of the human peripherin 5'UTR sequence replaced by mouse peripherin 5'UTR sequence) were cut with Bg1II and Avrll respectively, expressed *in vitro* and both RNAs mix d separately and together with Rib9 RNA to test for cleavage. RNAs were mixed

with varying concentrations of $MgCl_2$ to optimise cleavage of RNAs by Rib9^V RNA (Figures ~~16-19~~ 17-20). Notably the majority of the larger unadapted RNA product was cleaved while the adapted RNA product with altered non-coding sequence remained intact (Figures ~~16-19~~ 17-20). Similar results were obtained with Rib8^V RNA which targets a different open loop than Rib9^V RNA in the non-coding sequence of human peripherin. However in the case of Rib8^V RNA the extent of the cleavage was significantly less than Rib9^V RNA (Figures ~~16-19~~ 17-20) suggesting the important role of RNA structure in antisense binding and RNA cleavage.

Example 5

Human COL1A2

Rib18 which has been cloned into pCDNA3 (~~Sequence~~ SEQ ID NO: 16) targets the 5'UTR sequence of the human type I collagen COL1A2 gene, multiple mutations in which can cause autosomal dominantly inherited osteogenesis imperfecta involving bone fragility amongst other symptoms. A clone containing the 5'UTR sequence together with exon I of the human COLIA2 gene has also been generated (~~Sequence~~ SEQ ID NO: 15) to apply suppression and replacement strategies to this human gene.

Antisense constructs

A number of constructs have been generated in pCDNA3 and pZEOSV containing tracks of sense and antisense sequence from the non-coding regions of the mouse rhodopsin and peripherin genes. An example of these sequences is given in (~~Sequences~~ SEQ ID NOS: 17 and 18). Antisense effects are under evaluation.

DISCUSSION

In the first four examples outlined above, RNA was

expressed from cDNAs coding for four different proteins: mouse and human rhodopsin and mouse and human peripherin. All four RNAs have been significantly attacked *in vitro* using suppression effectors directed towards the non-coding regions of the RNA. In all four examples the ribozymes directed to 5'UTR sequences were successful in cleaving target RNAs in the predicted manner. Antisense targeting non-coding sequences was used successfully to elicit binding and cleavage of target RNAs in a sequence specific manner.

In some cases it is possible that cleavage of the RNA at the 5'UTR would not effect the functioning of the resulting RNA cleavage products in generating protein. Moreover although lowering RNA levels may often lead to a parallel lowering of protein levels this is not always the case. In some situations mechanisms may prevent a significant decrease in protein levels despite a substantial decrease in levels of RNA. However in many instances suppression at the RNA level has been shown to be effective (see prior art). In some cases it is thought that ribozymes elicit suppression not only by cleavage of RNA but also by an antisense effect due to the antisense arms in the ribozyme. Notably we have demonstrated sequence specific attack of target RNAs in non-coding regions, which is an important stage in gene suppression.

In the four examples provided ribozymes were designed to target 5'UTR sequences, however, they could be readily designed to target any non-coding sequences. Suppression could be achieved using antisense or ribozymes targeting for example, the 3'UTR sequences or any combination of non-coding sequences.

Additionally, in all four examples, cDNAs with altered

sequences in the non-coding regions targeted by ribozymes were generated. RNAs expressed from altered cDNAs were protected entirely from cleavage due the absence of the ribozyme target by each of the ribozymes tested. Alterations involved replacement of UTR sequence with UTR sequence from another gene expressed in the same tissue or UTR sequence from the same gene but from a different mammalian species (e.g. mouse peripherin, human peripherin, mouse rhodopsin). In one case the target site was deleted (human rhodopsin). Of particular interest is the second mouse rhodopsin hybrid cDNA for Rib3 which contains a single base change thereby preventing RNA cleavage. In some cases the non-coding sequences of a gene may be essential to the overall efficient expression and functioning of the gene. Therefore it may be useful to alter replacement genes in subtle ways to prevent ribozyme cleavage or nucleic acid binding. Changing a few nucleotides in many instances may be sufficient to prevent nucleolytic attack.

As highlighed before in this text using this invention the same method of suppression (targeting non-coding sequences) and gene replacement (using a gene with altered non-coding sequences) may be used as a therapeutic approach for any mutation within a given gene.

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CLAIMS

1. A strategy for suppressing expression of an endogenous gene, wherein said strategy comprises providing suppression effectors able to bind to the non-coding regions of a gene to be suppressed, to prevent the functional expression thereof.

2. A strategy as claimed in claim 1 wherein the suppression effectors are antisense nucleic acids.

3. A strategy as claimed in claim 1 or claim 2 wherein non-coding regions include the transcribed but non-translated regions of a gene.

4. A strategy as claimed in claim 2 or 3 wherein the antisense nucleic acids are DNA or RNA, directed to 5′ and/or 3′ untranslated regions and/or to introns and/or to control regions or to any combination of such untranslated regions.

5. A strategy as claimed in any of the preceding claims wherein strategy further employs ribozymes.

6. A strategy as claimed in any of the preceding claims wherein the strategy further employs nucleotides which form triple helix DNA.

7. A strategy as claimed in any of the preceding claims wherein the suppression effectors are incorporated into a vector.

8. A strategy as claimed in claim 7 wherein the vector is chose from DNA plasmid vectors, RNA or DNA virus vectors.

9. A strategy as claimed in claim 7 or 8 wherein the vector is combined with lipids, polymers or other derivatives.

10. The use of a strategy as claimed in any preceding claim in the preparation of a medicament for the treatment of an autosomal dominant disease.

11. A strategy as claimed in any of claims 1 to 9 wherein the gene includes promoter regions.

12. A strategy for suppressing an endogenous gene and introducing a replacement gene, said strategy comprising the steps of:

1. providing antisense nucleic acid able to bind to at least one non-coding or untranslated region of a gene to be suppressed and

2. providing genomic DNA or cDNA encoding a replacement gene sequence,

wherein the antisense nucleic acid is unable to bind to equivalent non-coding or untranslated regions in the genomic DNA or cDNA to prevent expression of the replacement gene sequence.

13. A strategy as claimed in claim 12 wherein control sequences of the replacement nucleic acid belong to a different mammalian species, a different human gene or are similar but altered from those in the gene to be suppressed and thus permit translation of the part of the replacement nucleic acid to be initiated.

14. Replacem nt nucleic acids for use in a strategy as

claimed in any of claims 1 to 9 or claims 11 to 13, with altered non-coding sequences such that replacement nucleic acids cannot be recognised by naturally occurring endogenous suppressors present in one or more individuals, animals or plants.

15. Replacement nucleic acids as claimed in claim 14 comprising altered non-coding sequences to provide the wild type or equivalent gene product being at least partially protected from suppression by naturally occurring endogenous suppression effectors.

16. The use of a vector or vectors containing suppression effectors in the form of nucleic acids, said nucleic acids being directed towards untranslated regions or control sequences of the target gene and vector(s) containing genomic DNA or cDNA encoding a replacement gene sequence to which nucleic acids for suppression are unable to bind, in the preparation of a combined medicament for the treatment of an autosomal dominant disease.

17. A method of treatment for a disease caused by an endogenous mutant gene, said method comprising sequential or concomitant introduction of (a) antisense nucleic acids to the non-coding regions of a gene to be suppressed; to the 5′ and/or 3′ untranslated regions of a gene or intronic regions or to the non-control regions of a gene to be suppressed, (b) replacement gene sequence with control sequences which allow it to be expressed.

18. A method of treatment as claimed in claim 17 wherein the nucleic acid for gene suppression is administered before or after or at the same time as

the replacement gene is administered.

19. A kit for use in the treatment of a disease caused by an endogenous mutation in a gene, the kit comprising nucleic acids for suppression able to bind to the 5′ and / or 3′ untranslated regions or intronic regions or control regions of the gene to be suppressed and a replacement nucleic acid to replace the mutant gene having a control sequence to allow it to be expressed.

20. A method of treatment as claimed in claim 17 or 18 wherein nucleotides can be administered as naked DNA or RNA, with or without ribozymes and/or with dendrimers.